MICROBIOLOGICAL PROBLEMS ASSOCIATED WITH THE DECOMPOSITION OF HUMIC ACID

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The organic matter in soil can be divided into organic debris, in which there is still clear evidence of the original structure of the leaves, etc., and humus, which is generally described as the black amorphous organic material remaining after primary decomposition has taken place. From early work it is clear that humus can be separated into a number of fractions. One of these is humic acid, which is usually obtained by extracting soil with alkalies and precipitating the crude humic acid by the addition of mineral acid. Extraction of the precipitate with alcohol removes hymatomelanic acid. The humic acid can be further purified by removing fatty material with ether, and mineral matter by dialysing against dilute hydrochloric acid. Using such methods, samples of humic acid can be obtained from most soils. Little is known at present regarding the formation, chemical structure, and decomposition of this material, nor of the other fractions present in humus. With our present knowledge of the chemistry of humic acid, it is not possible to say whether the products obtained by different extractants represent a single chemical substance, or even whether the extracts obtained from different soil types represent the same material. It is because of these doubts that the present investigation has been restricted to humic acid prepared from a single horizon of the podzol at Delamere Forest, and using a single extractant, the view being taken that because of the possibility of there being a number of different forms of humic acid, work should initially be confined to one form of humic acid. The B_1 horizon was used because it represents a relatively pure natural accumulation of humic acid. Although the present methods have worked well in this investigation, they are not necessarily applicable to all other soil types.

Method of extraction. In order to obtain consistent material for the microbiological work, humic acid has been extracted as follows:

Lactic acid is used as the extractant, as Dr. S. Hepple working in this department has shown that it provided an excellent extractant for humic acid from the B_1 horizon of a podzol. Extractants such as NaOH were avoided, since it is known that humic acid absorbs oxygen under alkaline conditions, and also the use of acetyl bromide and other such drastic treatments were considered undesirable. 50% (w/v) lactic acid is allowed to percolate through a column of the soil sample which is mixed with cleaned coarse quartz sand to assist drainage. The resulting clear brown solution is filtered through No. 3 Whatman filter-paper, and then passed through an ion-exchange column, containing amberlite resin I.R.120 to remove cations, principally iron. The solution is then diluted with about 4 parts distilled water, and concentrated hydrochloric acid is added until precipitation occurs. The precipitate is allowed to settle, the supernatant decanted off, and is then centrifuged and placed in vinyl dialysis tubing. It is dialysed against 20% (v/v) HCL until iron can no longer be detected. The tubes are then transferred to water, and dialysed until no further chlorine ions are present. The humic acid is dried in a vacuum oven at 40°. 1000 g. of fresh soil gives a yield of approximately 20 g. of dried humic acid. The dried product is dark brown in colour, and is an amorphous powder.

Chemical analyses have shown that the product obtained has an approximate composition as follows: C 50; H 3·9; O 38; N 0·3–0·4; OMe 1; ash, 1–2%.

The ash consists of 25% Si, 18% Fe_2O_3, 16% TiO_2, as well as more than 5% of Ca and of Na and traces of K, Mg, Cu, V, and Sr.

Enrichment experiments

In an attempt to obtain organisms capable of utilizing humic acid, a series of enrichment experiments have been carried out, using shake cultures in flasks, vermiculite in Petri dishes, and soil-perfusion techniques. The soil-perfusion apparatus used is that designed by G. Metcalfe (Naguib, 1957).

At intervals the culture vessels have been sampled and a comparison made between the organisms present in the controls and those in which humic acid had been added. In all these experiments there was an increase in the number of species of fungi and in their abundance when humic acid was present, but no particular organism or organisms have persistently dominated in the humic-acid cultures when compared with the controls, with the possible exception of *Trichoderma viride*.

Table 1 summarizes the information so far available from these enrichment experiments. The results shown under heading A in Table 1 are those from 4 soil perfusion experiments. Separate perfusion columns were prepared containing the 3 substrates used—soil, sterile sand, and sterile vermiculite, in each experiment, making a total of 12 columns with humic acid and 12 controls.

The four experiments were as follows:

(1) 8/11/56. Perfusion with Knop's solution, no carbohydrate, room temperature, inoculated with garden soil.
(2) 28/8/57. Perfusion with Czapek's solution, 0·5% (w/v) sucrose,

room temperature, inoculated with soil from mixed broad-leaf forest.

(3) 15/12/57. Perfusion with Czapek's solution, no carbohydrate, room temperature, inoculated with garden soil.

(4) 15/12/57. As in (3), but at 25°.

The humic acid was dissolved in 1% (w/v) K_3PO_4 and added to the perfusate to make a concentration of 0·1%, while K_3PO_4 alone was added to a similar series of controls.

TABLE 1

Frequency of stimulation or inhibition of fungi in presence of humic acid. Arranged in a decreasing sequence of stimulation

	Name of fungus	A Soil perfusion experiments			B Flask culture and vermiculite-plate experiments		
		Stimulation	No change	Inhibition	Stimulation	No change	Inhibition
Stimulated	*Candida* sp.	7	3	2	6	1	0
	Cephalosporium sp.	5	5	2	—	Absent	—
	Trichoderma viride	5	5	2	7	1	1
	Penicillium spp. monoverticilliate	4	4	2	6	2	0
	Other Penicillia	4	4	2	0	5	0
	Scopulariopsis spp.	2	1	0	—	Absent	—
	Fusarium spp.	1	2	1	9	1	0
	Mucor spp.	3	7	2	1	0	0
No change	Dematiaceae	2	9	1	—	Absent	—
	Verticillium sp.	2	8	2	—	Absent	—
	Actinomycetes	3	8	1	1	0	1
	Zygorrhynchus spp.	0	10	2	2	1	0
	Aspergillus spp.	1	8	3	—	Absent	—

The results from the flask-culture and vermiculite-plate experiments are shown under heading B in Table 1. 150-ml. flasks were used each containing 25 ml. 0·06% (v/v) Knop's solution, and were inoculated in one series with greenhouse soil and in the other with soil from a vegetable plot. There were three replications in each experiment, and each flask was subcultured by transferring a loopful to a similar fresh solution on 5 successive occasions at intervals of 2 weeks.

Another two series were inoculated with garden soil. In one series 0·06% of Knop's solution was used; in the other a medium containing:

$(NH_4)_2 SO_4$	0·1% (w/v)
$K_2 HPO_4$	0·1% (w/v)
$CaCO_3$	1·0% (w/v)
Tap water pH	6·4

The vermiculite plates were kept moist with these same two solutions in the two series, and were inoculated with 4 different garden soils. There were 5 replications in each experiment. In both the flasks and the plates humic acid was added to one set and the other run as a control set as in the perfusion experiment.

The results indicate the number of experiments in which the frequency of the particular organism was increased or decreased as compared with the control, which did not contain humic acid. Determination of frequency was by means of soil-crumb and dilution plates.

Growth of fungi on humic-acid media

Certain of the fungi which appear to be most frequently associated with the humic-acid cultures were selected for detailed study. The organisms chosen were *Paecilomyces* sp., *Penicillium spinulosum*, *Penicillium* sp., *Fusarium solani*, *Trichoderma viride*, *Scopulariopsis* sp., *Geotrichum candidans*, and *Candida* sp. These were tested on Czapek's mineral medium, omitting the sucrose, and with humic acid as the sole source of carbon. No satisfactory evidence of utilization of this substrate has been obtained. On plate cultures, growth is very sparse on both controls and those with humic acid. An increased linear spread was shown by some species, notably a *Paecilomyces* sp., and the results of an experiment with this organism are shown in Fig. 1. A similar sparse growth occurs in flask culture, and the dry weight of the mycelium of any of these fungi after 10 days never exceeded 5 mg. per 25 ml. medium in either control or test flasks. Although *Paecilomyces* showed an increased linear spread it gave no increased growth in flask culture.

In many experiments a supplement of sugar was added, and in some cases there appeared to be evidence of utilization, but careful investigation showed that these apparently successful results could be interpreted differently. Plates containing 0·1% (w/v) humic acid which were inoculated with *Penicillia* spp., *Scopulariopsis* spp., or with *Streptomyces* spp., showed a colourless zone around the growing colony where the dark brown colour of the humic acid had disappeared. This suggested a utilization of the humic acid. Similarly, if these fungi were grown in shake cultures in flasks containing variants of Czapek's medium and 0·1% (w/v) humic acid, the medium which was initially dark brown in

colour gradually became paler, and in the case of experiments with *Penicillium* (?) *oxalicum* in a medium with ammonia nitrogen replacing nitrate nitrogen, the colour completely disappeared after 6 days, again suggesting some breakdown and utilization of the humic acid.

Microscopical observation of the mycelium from such plate or flask cultures showed that they were stained a reddish-brown colour. Frequently this took the form of a thick sheath coating the cell wall and in media which develop a low pH, as with ammonia nitrogen, this sheath

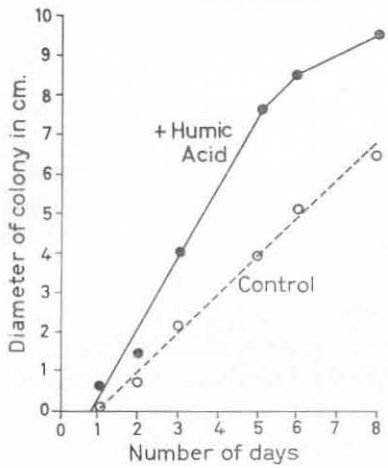

Fig. 1. Linear growth of Paecilomyces sp. on Czapeks Agar Plates.

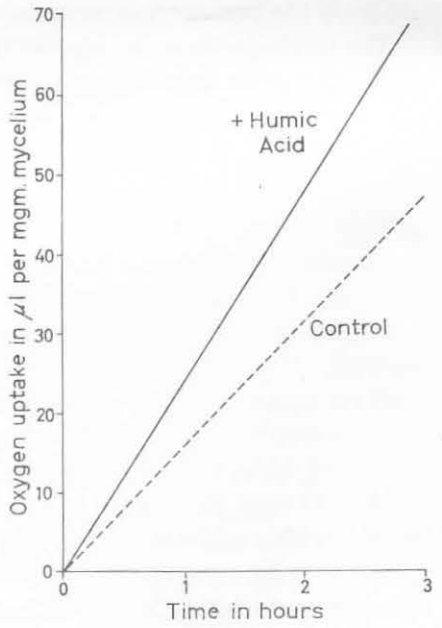

Fig. 2. Effect of Humic acid on the respiration of Paecilomyces sp.

may appear granular due to precipitation of humic acid on the mycelial walls. This unusual appearance of a sheath in humic-acid media has previously been described by Flaig & Schmidt, (1957). Stained mycelium was removed from the flasks and the pellets extracted with warm 1% (w/v) K_3PO_4 solution and this led to the recovery of the humic acid apparently unchanged. After precipitation and washing, the dry weight obtained showed that up to 93% could be re-extracted from the mycelium and the culture solution. The 7% loss could well be due to experimental error, since it is difficult to remove completely all traces of colour from the mycelium.

However, these results must be interpreted with reservation, since it

has also been shown that the nitrogen content of humic acid extracted from media and mycelium is increased from about 0·4% to 4·2%, presumably due to chemical combination of the humic acid with nitrogen compounds which is well known.

If this nitrogen is present as amino acids or protein, calculation shows that up to 24% of the original low-nitrogen humic acid could have been utilized. This confusion is greatly increased in media with organic nitrogen, and makes any quantative determination of humic acid after growth very difficult.

In parallel experiments, the disappearance of humic acid was checked by using a spectrophotometer, and this again indicated that the re-extracted humic acid was almost identical in amount and nature to that originally present before growth.

In further experiments to test whether there was an increase in dry weight of mycelium in the presence of humic acid compared with the control medium, a number of dry-weight experiments were undertaken and sucrose was added in varying amounts as a carbon supplement. The dry weight was determined on weighed sinter funnels. Allowance was made for the adhering humic acid, either by washing the harvested mycelium with potassium phosphate or by recovering the humic acid from the culture solution and obtaining, by calculation, the amount which could have been absorbed. No experiment has given unequivocable evidence of an increase in growth due to humic acid when supplied as the sole carbon source, nor when sucrose is provided as an initial carbon substrate.

Respiration of fungi in humic-acid media

Experiments in which humic acid was added to washed mycelial pellets in Warburg flasks and the resulting oxygen uptake measured have shown a marked effect of humic acid on some of the soil organisms. A typical experiment with *Paecilomyces* sp. gives approximately 30% increase of oxygen uptake in the presence of humic acid, as shown in Fig. 2. Other fungi which gave a similar result include *Scopulariopsis* sp. and *Asteromyces cruciatus*. A detailed consideration of these results, coupled with those from the growth rate experiments, leads us to conclude that the oxygen uptake recorded does not necessarily represent increased respiration due to utilization of the humic acid, but is more akin to the decoupling effect as obtained with dinitro-phenol.

The above experiments are recorded in detail because they illustrate the difficulties and problems associated with the microbiological investigations of the utilization of humic acid, and also suggest that in

many of the experiments previously recorded in the literature, the evidence is not sufficiently conclusive to warrant the claim that the substrates tested have been utilized by the fungi under investigation.

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